Pre-clinical characterization of signal transducer and activator of transcription 3 small molecule inhibitors for primary and metastatic brain cancer therapy


HHA, CP, NV, JS, RD, PRL, MGC: Department of Neurosurgery and Department of Cell and Developmental Biology, University of Michigan School of Medicine, MSRBII Rm 4570, 1150 Medical Center Drive, Ann Arbor, MI

HHA: Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, UCLA, Los Angeles, CA

EP, JH: Department of Chemistry, Eastern Michigan University, Ypsilanti, Michigan

HA: Department of Pathology, The University of Michigan School of Medicine, University Hospital, Ann Arbor, Michigan, USA

LR: Department of Biomedical and Molecular Sciences, Queen’s University School of Medicine, Botterel Hall Rm 713, Ontario, Canada

TM: Department of Neurology, Henry Ford Hospital, Detroit, Michigan
Running Title: Chemotherapy for glioma and melanoma

Corresponding author: M.G. Castro, PhD

Address: MSRB II, Room 4570
University of Michigan School of Medicine
1150 West Medical Center Drive
Ann Arbor, MI 48109-5689

Telephone: 734-764-0850
Fax: 734-764-7051
E-mail: mariacas@umich.edu

Total number of pages: 35
Tables: 0
Figures: 6
References: 50
Words in Abstract: 246
Words in Introduction: 698
Words in Discussion: 1,136
Supplementary Materials: Materials and Methods, Tables: 2, and Figures: 9

List of non-standard abbreviations: AG490, (E)-2-Cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide; AZD1480, 5-Chloro-N2-[(1S)-1-(5-fluoro-2-pyrimidinyl)ethyl]-N4-(5-methyl-1H-pyrazol-3-yl)-2,4-pyrimidinediamine; BBB, blood-brain barrier; Bcl-xl, B-cell lymphoma-extra large; B16-f0 or B16-f10, Mouse melanoma cell lines; B16EGFRvIII, B16
murine melanoma cells overexpressing epidermal growth factor vIII; CNS, Central nervous system; CNS-1, Rat glioma cell line; CPA-7, trichloronitritodiammineplatinum(IV), fac-[Pt(NH₃)₂Cl₃NO₂]; ML116, 4-benzyl-1-{thieno[2,3-d]pyrimidin-4-yl}piperidine, C₁₃H₁₉N₃S; CT26, Mouse colon carcinoma cell line; C57BL/6, common inbred strain of laboratory mouse; EGF, epidermal growth factor; FGF, fibroblast growth factor; FLLL32, (2E,2'E)-1,1'-(cyclohexane-1,1-diyl)bis(3-(3,4-dimethoxyphenyl)prop-2-en-1-one); GBM, glioblastoma multiforme; GL26, Murine glioma cell line; HEK293, Human embryonic kidney cell line; HF2303, Primary cultured glioma tumor cell line; IC₅₀, half maximal inhibitory concentration; IFNγ, Interferon gamma; IHC, Immunohistochemistry; IL-1β, Interleukin-1β; IL-6, interleukin 6; IN859, Human glioblastoma cell line; ISRE, Interferon-Stimulated Response Element; JAK, Janus Kinase; LLL12, 5-hydroxy-9,10-dioxo-9,10-dihydroanthracene-1-sulfonamide; MDA-MB-231, human breast carcinoma cell line; MG-132, Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; N9, Immortal mouse microglial cell line; PAMPA, parallel artificial membrane permeability assay; Pₐₑffective, Effective permeability; PI, Propidium Iodide; SAR302503, N-tert-Butyl-3-\{5-methyl-2-[4-(2-pyrrolidin-1-yl-ethoxy)-phenylamino]-pyrimidin-4-ylamino\}-benzenesulfonamide; SEI, Sis-inducible element; SMA560, Murine glioma cell line; STAT1, Signal transducer and activator of transcription 1; STAT3, Signal transducer and activator of transcription; STAT5 Signal transducer and activator of transcription 5; Src, proto-oncogene c-Src [sarcoma]; TNFα, Tumor necrotic factor α; U251, Human glioblastoma cell line; WP1066, (S,E)-3-(6-Bromopyridin-2-yl)-2-cyano-N-(1-phenylethyl) acrylamide, C₁₇H₁₄BrN₃O.

Section Assignment: Chemotherapy, Antibiotics, and Gene Therapy
Abstract

Signal transducer and activator of transcription 3 (STAT3) has been implicated as a hub for multiple oncogenic pathways. The constitutive activation of STAT3 is present in several cancers including gliomas (GBMs) and is associated with poor therapeutic responses. Phosphorylation of STAT3 triggers its dimerization and nuclear transport where it promotes the transcription of genes that stimulate tumor growth. In light of this role, inhibitors of the STAT3 pathway are attractive therapeutic targets for cancer. To this end, we evaluated the STAT3 inhibitory activities of three compounds (CPA-7, WP1066, and ML116) in cultured rodent and human glioma cells, including GBM cancer stem cells. Our results demonstrate a potent induction of growth arrest in GBM cells after drug treatment with a concomitant induction of cell death. Although these compounds were effective at inhibiting STAT3 phosphorylation, they also displayed variable dose-dependent inhibition of STAT1, STAT5, and NF-κβ. The therapeutic efficacy of these compounds was further evaluated in peripheral and intracranial mouse tumor models. While CPA-7 elicited regression of peripheral tumors, both melanoma and GBM, its efficacy was not evident when the tumors were implanted within the brain. Our data suggests poor permeability of this compound to tumors located within the central nervous system. WP1066 and ML116 exhibited poor in vivo efficacy. In summary, CPA-7 constitutes a powerful anti-cancer agent in models of peripheral solid cancers. Our data strongly supports further development of CPA-7-derived compounds with increased permeability in order to enhance their efficacy in primary and metastatic brain tumors.
Introduction

Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family of proteins whose main function is to relay signals from a specific set of receptor tyrosine kinases and cytoplasmic non-receptor tyrosine kinases to the nucleus where gene transcription takes place (Groner et al., 2008). Although the biological activities of STAT proteins vary, STAT3 has gained notoriety as a signaling hub for multiple oncogenic pathways (Yu et al., 2007, Al Zaid Siddiquee et al., 2008, Brantley et al., 2008). Constitutive activation of the STAT3 pathway has been noted several cancer types and typically occurs in response to stimulation by tumor promoting factors, i.e., EGF, FGF, IL-6, Src, amongst many others (Zhong et al., 1994, Migone et al., 1995, Yu et al., 1995, Bromberg et al., 1998, Abou-Ghazal et al., 2008, Srinivasan et al., 2008). STAT3 activation is mediated through binding to specific transmembrane STAT3/STAT3-dependent receptors. STAT3 becomes activated by JAK-dependent Tyrosine phosphorylation on a critical Tyrosine residue (Tyr705), dimerizing through reciprocal Src homology 2-phosphotyrosine interaction (Prinz et al., 2011). After dimerization, STAT3 translocates to the nucleus and binds to specific sequences within promoter regions, inducing gene transcription. STAT3 contains a second phosphorylation site (Ser727) within its C-terminal region; Ser727 phosphorylation is a secondary event after Tyr705 phosphorylation; which is required for the maximal transcriptional activity of STAT3. Constitutive activation of STAT3 shown by phosphorylation of the Tyr705 residue is present in several types of cancers, including melanomas and gliomas (Niu et al., 2002, Hussain et al., 2007, Kong et al., 2008). Phosphorylation of STAT3 results in the transcription of genes that promote cell proliferation, survival, vascularization, and immunosuppression (Chen et al., 2000, Yu et al., 2007). Inhibition of STAT3 activity in experimental tumor models has provided clear evidence for the role of
STAT3 signaling in the growth of tumors; thus becoming an attractive target for cancer therapy (Turkson et al., 2000, Stechishin et al., 2013).

Small molecule inhibitors of STAT3 activation have been intensely pursued, i.e., Sorafenib, a multi-kinase inhibitor approved for use in advanced renal cell carcinoma and hepatocellular carcinoma patients, has been shown to reduce STAT3 activation (Yang et al., 2008). The preclinical compounds LLL12 and FLLL32, analogues of curcumin, were also effective inhibitors of STAT3 activation (Wei et al., 2011). In addition to down-regulating STAT3 activity in human rhabdomyosarcoma cells, these compounds were effective in reducing the growth of MDA-MB-231 breast cancer xenografts in mice (Lin et al., 2010, Wei et al., 2011). However, these are indirect inhibitors of STAT3 as they act by inhibiting the upstream Janus kinase 2 (JAK2). Recent clinical trials investigating the use of the JAK1/2 inhibitor AZD1480 were terminated in phase I when it was reported that the majority of patients experienced adverse events or dose-limiting toxicities (Plimack et al., 2013).

In this study, we evaluated three compounds (CPA-7, WP1066, and ML116) as inhibitors of STAT3 activation in mouse models of glioma and melanoma. CPA-7 is considered an analogue of cisplatin, whose derivatives exhibit variable effects on DNA structure and signal transduction (Boulikas et al., 2003). Of these platinum complex derivatives, CPA-7 has been shown to preferentially target STAT3 in transformed cells with potent tumoricidal activity (Littlefield et al., 2008, Zhang et al., 2009). Inhibition of growth in subcutaneous CT26 mouse tumors after CPA-7 administration has also been reported (Turkson et al., 2004). STAT3 inhibition in tumor cells has been shown to up-regulate pro-inflammatory cytokines, activating
innate immune responses, and dendritic cells, resulting in potent antitumor T-cell responses (Wang et al., 2004). CPA-7 has also been shown to increase IL-1β and TNFα in N9 microglia, implicating STAT3 inhibition in the modulation of the microglial-mediated immune response (Zhang et al., 2009).

WP1066 is an analogue of a previously identified compound (AG490) and has been shown to block STAT3 activation by inhibition of the upstream JAK2 signaling (Iwamaru et al., 2007, Kong et al., 2008). We also evaluated ML116, which had recently been identified in a high-throughput screen as a specific and potent inhibitor of STAT3 (Madoux et al., 2010).

Although these compounds have been previously shown to inhibit STAT3 activation in certain transformed cells, their specificity and in vivo efficacy has not been thoroughly evaluated (Hussain et al., 2007, Zhang et al., 2009, Madoux et al., 2010). In this study, we report the tumoricidal activity and specificity of these compounds in a panel of GBM lines, primary GBM cells, and human glioma cells with stem cell properties (Ausman et al., 1970, Sampson et al., 1997, Candolfi et al., 2007, Kumar et al., 2012). In addition, the therapeutic efficacy of CPA-7, WP1066, and ML116 was evaluated in mice bearing intracranial or peripheral GBM (GL26), and melanoma (B16-f0 or B16-f10) tumors. While the compounds were effective at inducing the death of cancer cells in vitro, ML116 and WP1066 were poor at inhibiting growth of intracranial and peripheral tumors in vivo. Conversely, CPA-7 administration induced tumor regression in mice bearing subcutaneous tumors but was ineffective if the tumors were located within the brain. This differential activity of CPA-7 was attributed to poor blood-brain barrier (BBB)
permeability, which was confirmed using IHC, western blot, and parallel artificial membrane permeability assay (PAMPA).
Materials and Methods

Cell lines, antibodies, and reagents.

Cultured cells were obtained from the following vendors: GL26 (ATCC, Manassas, VA), HEK-293 (Microbix, Toronto, CA), B16-f0 (ATCC® CRL-6322). HEK293 cells were cultured in MEM with supplementation (non-essential amino acids, 1% Pen-Strep, 1% L-Glutamine 10% Fetal Bovine Serum). All other glioma cells were grown in DMEM with supplements. HF2303 (sex unknown) were grown in neurosphere conditions which consisted of DMEM/F12 50:50 media supplemented with N-2 supplement, and 1% Pen-Strep, and a 1% Anti-fungal in addition to having 10 ng/mL rEGF and rFGF. Luciferase reporter plasmids were purchased from Promega (Madison, WI). Antibodies for pSTAT3, STAT3, cyclin D, and Mcl-1 were purchased from Cell Signaling (Danvers, MA, Cat#9145, #9149, #2922, #5453). Anti-Bcl-xl was purchased from Abcam (Cambridge, England, Cat#ab2568). Survivin was ordered through Santa Cruz Biotechnologies (Dallas, TX, Cat#sc-8808), and anti-actin antibody was purchased from Sigma (St. Louis, MO, Cat#A1978).

Synthesis, administration, and validation of STAT3 small molecule inhibitors.

Methods of synthesis, administration, and validation of small molecule inhibitors can be found in supplementary materials and methods (Supplementary Table 2, Supplementary Figure 8, and Supplementary Figure 9).

Annexin V / PI flow cytometry

Glioma cells were seeded onto 12 well plates for apoptosis studies. STAT3 inhibitor was added to the cells after they were fully adhered and incubated for 48 hours before processing. Annexin
V/PI staining kit was purchased from Invitrogen. Floating and adherent cells were collected in 5 mL FACS tubes for staining using the manufacturer’s suggested protocol. All flow data was collected on a FACS ARIA II cell sorter (BD) and analyzed with Summit software (Dako) or flowjo software (Treestar inc).

**Tumor models**

GL26 glioma cells were originally derived from a female C57BL/6J mouse bearing a chemically induced intracranial neoplasm and used to generate syngeneic mouse glioma models (Ausman et al., 1970). B16-f0 and its more invasive derivative B16-f10 originated as melanoma cell lines used in this study as alternative models of GBM (Fidler 1973, Raz et al., 1980). Intracranial implantation of tumor cells was performed as previously described (Assi et al., 2013). A brief description of procedures for intracranial and flank tumor models can be found in supplementary materials and methods. All animal experiments conformed to the policies and procedures of the University’s Committee on Use and Care of Animals (UCUCA) and Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan. Mice used in this study were monitored and euthanized at the first signs of moribund behavior.

**Paraffin IHC**

A detailed methodology of immunohistochemistry can be found in supplementary material and methods.

**In vitro proliferation assay**

Proliferation of glioma cells was determined by measuring ATP levels using the CellTiter-Glo® Luminescent Cell Viability Assay provided by Promega, Cat# G7572 (Madison, WI). 1,000 cells
were plated in 100 µL of media and allowed to adhere for 24 hours before the addition of CPA-7, ML116, or WP1066. ATP levels in 96-wells were measured each day according to manufactures’ instructions (Promega).

Western Blot

Whole cell lysates were prepared with RIPA buffer supplemented with protease and phosphatase inhibitors as described previously (Curtin et al., 2009) A more detailed explanation of western blotting can be found in supplementary materials.

Luciferase assay for transcriptional activity

Transcriptional activity was assessed in stable U251 reporter clones. A detailed description of the process by which reporter cells were generated and utilized for readout of transcriptional activity can be found in supplementary materials and methods.

PAMPA assay

PAMPA assay was performing according to protocols outlined by the manufacturer Pion, Inc (Billerica, MA) in accordance with the Vahlteich Medicinal Chemistry Core at the University of Michigan. Permeability assays were performed at a pH of 7.4. Diffusion of compounds across the lipid layer was measured after a 4 hour incubation period at room temperature.

STATISTICAL ANALYSIS

Error bars represent SEM and asterisks denote statistically significant results. P-values of less than 0.05 were considered significant, unless noted otherwise. Statistical significance was
calculated using NCSS (NCSS LLC, Kaysville, UT) or Graphpad software (Graphpad Software, Inc., La Jolla, CA). Dose-response curves and proliferation assays were analyzed by 2-way ANOVA followed by Tukey-Kramer multiple-comparison test was performed using NCSS. Apoptosis experiments and luciferase assays were analyzed using one-way ANOVA followed by Tukey’s Test to determine level of significance (Graphpad). Kaplan-Meier survival curves were analyzed using the Mantel log-rank test was used to determine statistical significance in median survival (Graphpad). A one-tailed student t-test was used to compare p-STAT3 intensity from western blots of flank and brain tumors treated with CPA-7 (Graphpad).
Results

Phosphorylated STAT3 is differentially expressed in cancer cells

To better understand the extent of STAT3 activation in GBM, we first screened a panel of glioma cell lines of mouse (GL26, SMA560), rat (CNS-1), and human (U251, IN859, HF2303) origin for phosphorylated STAT3 and total STAT3 levels by western blot (Supplementary Figure 1). For comparative purposes, whole cell lysates from HEK293 cells and two mouse melanoma variants, B16-f0 and B16-f10, were also included. Immunoblotting was performed using an antibody that recognizes the phosphorylated tyrosine705 residue of STAT3 (pSTAT3). Glioma cells harbored variable levels of STAT3 expression; GL26, U251, and IN859 cells contained the most abundant levels of pSTAT3. Primary human GBM cells cultured in neurosphere conditions were also positive for pSTAT3 expression (HF2303). In contrast CNS-1, SMA560, and B16-f10 cells had relatively low levels of pSTAT3 similar to HEK293 cells. The B16-f0 cell line showed the lowest levels of pSTAT3, only detectable after a very long exposure time (B16 Hi exp.; Supplementary Figure 1).

Inhibition of STAT3 signaling in glioma cells leads to an arrest of cell growth with concomitant induction of apoptosis

To determine the effects of STAT3 inhibition on cell proliferation in vitro, glioma cells were seeded in 96 well plates and treated the following day with CPA-7, WP1066, or ML116 at drug concentration doses ranging from 100 nM to 100 µM. After 48 hours, intracellular ATP levels were measured and plotted versus drug concentration to obtain dose-response data. All three compounds had a significant effect at inhibiting cell growth in a dose-dependent fashion (*, p < 0.05 versus vehicle; Figure 1A). Barring SMA560 and U251 cells, WP1066 appeared to be
the most potent with an IC$_{50}$ of 3-5 μM, depending on the cell type (Figure 1B). CPA-7 was also very effective at inhibiting the growth of glioma cells, albeit at a slightly higher concentration (IC$_{50}$: ~3-24 μM). While ML116 did block the proliferation of tumor cells, it was the least effective of the three compounds, requiring higher doses (~1- >50 μM) to elicit comparable effects to that of CPA-7 and WP1066. Using a single dose identified in the dose-response study we cultured glioma cells in the presence or absence of STAT3 inhibitors and assayed their proliferation over the course of 5-6 days. The amount of intracellular ATP was measured daily and plotted against time; vehicle treated cells were used as controls. CPA-7 and WP1066 were effective at inhibiting tumor cell growth with high efficacy in vitro (*, $p < 0.05$ versus vehicle; Figure 1C). Conversely ML116 was not as effective at inhibiting the proliferation of all cells tested (Figure 1C). The inhibition of tumor cell growth encountered lends support to the role of STAT3 as a growth-promoting factor in glioma and melanoma cells.

STAT3 is also known to mediate the transcription of genes that regulate apoptosis (Epling-Burnette et al., 2001; Gritsko et al., 2006). To determine if STAT3 prevents the induction of apoptosis in transformed cells, multiple glioma cell lines were treated with STAT3 inhibitors for 24 hours, after which flow cytometric analysis for Annexin-V binding and propidium iodide (PI) retention were performed. This technique permits the visualization of cells in the early (Annexin-V$^+$) and later stages of apoptosis or necrosis (PI$^+$ and Annexin-V$^+$). Cells treated with CPA-7 or WP1066 underwent complete apoptosis/necrosis within 24-48 hours while ML116 was less effective at inducing apoptotic cell death in tumor cells, as indicated by flow cytometric analysis (*, $p < 0.05$ versus vehicle; Figure 2).
Western blot analysis of STAT3 phosphorylation and its downstream targets in tumor cells treated with CPA-7, WP1066, and ML116

To better understand the mechanisms by which glioma cells are killed in response to CPA-7, WP1066, or ML116 whole cell lysates of treated tumor cells were probed by western blot for pSTAT3, total STAT3, and downstream transcriptional targets: Bcl-xl, survivin, and cyclin D1. The expression of STAT3 and its downstream target genes was evaluated in GL26, SMA560, and CNS-1 rodent glioma cells. CPA-7 or WP1066 were added to the cells at various doses for 24 hours. CPA-7 treatment elicited a dramatic reduction in the levels of pTyr705STAT3 in all tumor cells tested (Figure 3A). CPA-7 treated glioma cells were also found to have reduced levels of the anti-apoptotic genes Bcl-xl and survivin. The expression of the apoptosis inhibitor Bcl-xl is also STAT3-mediated, with Bcl-xl blocking apoptosis by preventing the release of cytochrome C from mitochondria and subsequently activating caspase (Kharbanda et al., 1997). Survivin can also inhibit apoptosis by binding activated caspase-3 and caspase-7 (Tamm et al., 1998). Cyclin D1, a STAT3 target gene involved in cell cycle progression, was also reduced after CPA-7 treatment demonstrating a considerable specificity for CPA-7. While WP1066 was also very effective at eliciting the apoptosis of glioma cells, this effect appeared to be less dependent on STAT3. This is best illustrated in GL26 cells where the addition of WP1066 has no impact on STAT3 phosphorylation or its downstream targets in spite of a strong induction of apoptosis (Figure 3B). SMA560 cells were also unique in that pSTAT3 levels actually rose with increasing doses of WP1066 before rapidly diminishing at 6 µM with negligible changes in the expression of Bcl-xl, surviving, or cyclin D. Unlike GL26 and SMA560 cells, the phosphorylation of STAT3 in rat CNS-1 cells was reduced following WP1066 treatment.
Human U251 GBM cells or primary human GBM neurospheres (HF2303) were also sensitive to STAT3 inhibition suggesting broad activity of CPA-7 and WP1066 across species (Figure 3C). ML116 did, in fact, inhibit STAT3 phosphorylation in U251 cells but at much higher concentrations (i.e., 100 µM) (Supplementary Figure 2). These results illustrate the changes in STAT3 phosphorylation and downstream proteins elicited by WP1066 and CPA-7. Moreover, our data show that WP1066 can exert anti-tumor activity independently of STAT3 in rodent GBM cells (Gl26).

**Transcriptional specificity of STAT3 inhibitors**

To determine the specificity of our compounds, transcriptional activity by STAT3, NF-κβ, STAT5, or STAT1 was assessed in U251 reporter cells, which we genetically engineered to stably express luciferase under the control of promoter regions sis-inducible element (SEI), NF-κβ, STAT5-RE, Interferon-Stimulated Response Element (ISRE), respectively. Transcriptional activity in response to the test compounds was then determined by measuring the bioluminescent signal produced by the cells after drug treatment. Effects on cell viability were factored in to reach a normalized value. CPA-7 and WP1066 were effective at inhibiting the transcriptional activity of STAT3 (*, p < 0.05 versus DMSO; Figure 4A). ML116 also had an effect on STAT3 activity, although this required the use of much higher doses (100 µM). At basal conditions NF-κβ and STAT1 transcriptional activity was low and necessitated the use of exogenous stimuli such LPS or IFNγ (†, p < 0.05 versus DMSO; Figure 4B). Pimozide, MG-132, and nifuroxazide were used as positive control inhibitors of STAT5, NF-κβ, and STAT1 respectively. NF-κβ-dependent activity, which can modulate STAT3 signaling by secretion of IL-6, was reduced in
response to CPA-7 and WP1066 (*, $p < 0.05$ versus LPS; Figure 4B). STAT5 activity appeared to be modulated in response to WP1066 at doses of 10 µM (*, $p < 0.05$ versus DMSO; Figure 4C). At very high doses (i.e., 100 µM), CPA-7 exhibited off-target effects and inhibited the activity of IFNγ-stimulated STAT1 (Turkson et al., 2004) (*, $p < 0.05$ versus IFNγ; Figure 4D). WP1066 also inhibited STAT1 activity, albeit at relatively low doses (3 µM and 10 µM).

**Therapeutic efficacy of CPA-7, WP1066, and ML116 in intracranial and peripheral mouse tumor models**

To generate intracranial tumors, GL26 cells were injected stereotactically into the striatum of syngeneic C57BL6/J. Animals harboring brain tumors were treated with CPA-7 as depicted in Figures 5A, B, C, D and euthanized at the first sign of morbidity. Intravenous delivery of CPA-7 into mice bearing intracranial GL26 tumors failed to extend the median survival as mice became moribund at the same time as vehicle treated animals (Figure 5B). In mature vertebrates, the BBB can impede the penetration of certain drugs into the central nervous system; therefore in addition to the intracranial model, we also tested CPA-7 in a peripheral subcutaneous GL26 tumor model. An identical treatment regimen was performed in mice harboring peripheral GL26 tumors (Figures 5E, F, G, H). In contrast to the intracranial model, mice bearing GL26 flank tumors treated with CPA-7 underwent complete tumor regression (*, $p < 0.05$; Figure 5F). In order to fully understand and characterize the safety features of this compound and before clinical implementation, full toxicology assessment is warranted.

The tumoricidal efficacy of WP1066 and ML116 was also evaluated in the syngeneic GL26 model, but we did not observe any statistically significant difference in the growth of brain or flank tumors (Supplementary Figure 3).
CPA-7 shares structural motifs with cisplatin, which is known to have restricted brain permeability. Given its highly polar structure, we hypothesized that diffusion of CPA-7 into brain tissue would also be limited, justifying its poor therapeutic efficacy in intracranial tumor models. To examine this, C57BL6 mice bearing either brain or flank tumors (GL26) were treated with CPA-7 for 7 days then terminally perfused and fixed for immunohistochemistry (IHC). To investigate CPA-7 penetrance and efficacy, tumors were either embedded in paraffin, sectioned, and immunolabeled for pSTAT3 (Figures 5C, G), or were harvested for western blot analysis of whole tumor lysate (Figures 5D, H). GL26 brain tumor-bearing mice treated with CPA-7 had no appreciable difference in levels of STAT3 phosphorylation (Figure 5C). In contrast, flank tumor bearing mice treated with STAT3 had reduced levels of pSTAT3 compared to vehicle treated controls especially at the tumor borders (Figure 5G). To further corroborate the IHC data, relative levels of pSTAT3 and total STAT3 were analyzed from whole tumor lysates by western blot. Immunoblot analysis of brain tumors treated with CPA-7 corroborated our findings from the IHC, showing no appreciable difference in the expression or phosphorylation of STAT3 in brain tumor lysates (Figure 5D), while flank tumors treated with CPA-7 showed a decrease of pSTAT3 and an associated decrease of total STAT3 as previously demonstrated in vitro (*, \( p < 0.05 \); one-tailed student t test; Figure 5H).

Parallel artificial membrane permeability assay (PAMPA) is a method used to assess the passive permeability of compounds across an artificial lipid membrane and has been found to correlate with BBB permeability (Avdeef 2005). As standards for comparison with the STAT3 inhibitors, the effective permeability (P_{eff}) of three control compounds, characterized by their
high (verapamil: $P_{\text{eff}} = 59.5 \times 10^{-6}$ cm/s), medium (cortisone: $P_{\text{eff}} = 23 \times 10^{-6}$ cm/s), and low (theophylline: $P_{\text{eff}} = 2 \times 10^{-6}$ cm/s) permeability, were derived simultaneously. WP1066 exhibited high permeability in the PAMPA assay with a $P_{\text{eff}}$ value of $54.6 \times 10^{-6}$ cm/s. Conversely the permeability of CPA-7 and ML116 were low with $P_{\text{eff}}$ values of $5.1 \times 10^{-6}$ cm/s and $3.7 \times 10^{-6}$ cm/s, respectively (Supplementary Figure 4). The poor permeability of CPA-7 observed in the PAMPA lends further support to the notion that this particular compound is ineffective for brain tumor models, as it cannot penetrate the blood brain barrier. ML116 shows a similar pattern of poor permeability as CPA-7, while WP1066 shows a high level of permeability in the PAMPA system.

**Therapeutic efficacy and toxicity of CPA-7, WP1066, and ML116 in melanoma models.**

The therapeutic efficacy of CPA-7, WP1066, and ML116 was also evaluated in the B16-f0 melanoma cell line and its B16-f10 derivative, a more invasive and metastatic clone. These cells form rapidly growing tumors 4-5 days after subcutaneous injection and are prone to ulceration around day 20. The cells can also be used to generate intracranial melanomas. As we observed in the GL26 model, administration of CPA-7 lead to an inhibition of tumor growth in both the B16-f10 and the B16-f0 flank tumors (*, $p < 0.05$ versus vehicle; Figure 6A), but had no therapeutic activity in mice bearing intracranial melanomas (Supplementary Figure 5). The lack of complete tumor regression in the B16 flank tumor models was attributed to the short therapeutic window as regressing tumor masses became ulcerated and had to be euthanized. No notable toxicity was featured in the liver (Figure 6B) or the spleens (Figure 6C) in any of the CPA-7-treated animals. We did observe moderate inflammation in the kidneys of CPA-7 treated mice, indicating tissue regeneration in response to a previous injury (Supplementary Figure 6).
A hematological and serum biochemical analysis (Supplementary Table 1) revealed a depression in several parameters from this cohort, including red blood cells, reticulocytes, white blood cells, lymphocytes, and monocytes (Figure 6D).

Administration of WP1066 had a small therapeutic effect in the B16-f0 flank tumor model which did not occur in B16-f10 flank tumor-bearing mice (Figure 6A). Also, splenic toxicity, characterized by an obliteration of the sinusoids by infiltration of transformed lymphocytes (Figure 6C), was also evident with WP1066 treatment. Finally, ML116 was the least efficacious of all three compounds, having no discernible therapeutic activity in either intracranial or flank the melanoma models (Supplementary Figures 5, 7, respectively).
Discussion

Constitutively activated STAT3 has been detected in the majority of advanced stage cancers including GBM and is generally correlated with poor prognosis (Abou-Ghazal et al., 2008, Morikawa et al., 2011). STAT3 signaling serves to promote the survival and proliferation of transformed cells by transcriptional regulation of anti-apoptotic and cell cycle genes (Brantley et al., 2008). In this study, we evaluated the in vitro specificity and tumoricidal activity of three STAT3 signaling inhibitors. In addition, the in vivo therapeutic activity and toxicity of these compounds was assessed in both intracranial and peripheral GL26, B16-f0, and B16-f10 mouse tumor models. Our results demonstrate an inhibition of glioma cells' proliferation in response to CPA-7, WP1066, and ML116. Effects of growth inhibition were associated with an induction of apoptosis in the majority of treated cells. Western blot analysis revealed the highly specific inhibition of STAT3 by CPA-7 in various glioma cell lines. This argument was strengthened by the inhibition of downstream targets of STAT3, including Bcl-xl, survivin, and cyclin D1. In contrast, western blot analysis of glioma cells treated with WP1066 alluded to a mechanism independent of STAT3 inhibition, as treatment with escalating doses of the compound showed no consistent decrease in pSTAT3 expression or its downstream targets. Using luciferase reporter assays our results demonstrate inhibition of STAT1 and STAT5 by WP1066. Therefore WP1066 acts as a JAK2 inhibitor, an observation that has also been noted by other groups (Iwamaru et al., 2007, Kong et al., 2008). We also demonstrate the inhibition of STAT1 in response to high concentrations of CPA-7, which is in agreement with previously published findings (Turkson et al., 2004).
While WP1066 appeared to exhibit potent cytotoxic activity in vitro, inhibition of STAT3 was only observed in a subset of these cell lines. This phenomenon was best illustrated in GL26 where addition of WP1066 had no impact on the phosphorylation status of STAT3 despite a robust induction of apoptosis. SMA560 cells were also unique in that pSTAT3 levels actually rose with increasing doses of WP1066 before rapidly declining at a concentration of 6 µM with negligible changes in the expression of cyclin D or Bcl-xl. In addition, WP1066 elicited significant off-target activity. The off-target effects observed in NF-κβ, STAT5, and STAT1 reporter cells suggest that WP1066 is a promiscuous inhibitor. A recent study which used a panel of 368 human kinases (covering ~60% of the human kinome) to profile the specificity of the JAK2 inhibitors ruxolitinib and SAR302503, revealed an inhibition of >30 kinases for ruxolitinib and >50 kinases for SAR302503 (Zhou et al., 2013).

WP1066 has been previously shown to elicit tumor regression in intracerebral B16EGFRvIII and B16-f0 tumor models when used at a dose of 40 mg/kg (Kong et al., 2008). When tested at a dose of 30 mg/kg in an intracerebral B16 melanoma model, this compound did not show therapeutic efficacy (Kong et al., 2008, Hatiboglu et al., 2012). Our results show that this compound was only mildly effective in B16-f10 flank tumors with negligible effects in GL26 or B16-f0 models, both intracerebral and flank models. The lack of therapeutic efficacy exhibited by WP1066 in our intracranial tumor models cannot be explained by poor BBB permeability; both our PAMPA results and data shown previously indicate that this compound exhibits good diffusion throughout the brain after systemic delivery (Hussain et al., 2007). WP1066 has been approved for a phase I clinical trial in patients with recurrent GBM or...
metastatic melanoma that will assess the maximum tolerable dose of WP1066 and monitor for any adverse events or dose-limiting toxicities.

CPA-7, on the other hand, elicits high therapeutic efficacy in peripheral tumor models (Figure 5F, 6A), in addition to high specificity for inhibiting STAT3-mediated signaling (Figures 3A, 4). Thus, CPA-7 is a strong candidate for further development as a novel therapeutic strategy for solid cancers. Although CPA-7 does not appear to penetrate the BBB as would be required to treat tumor located within the central nervous system, alternative therapeutic strategies, such as nanoparticles loaded with CPA-7, could serve as an effective delivery platform. Ultimately, the in vivo inhibition of tumor growth dictates the usefulness of these compounds as anti-cancer agents. In addition, to avoid toxicity associated with off-target effects, the compounds must be specific to their intended target.

The specificity of CPA-7 for its STAT3 target, as well as its potent anti-tumor effects in animals harboring peripheral GL26, B16-f0, or B16-f10 tumors that underwent complete tumor regression highlight its promise as a novel anti-cancer agent. The lack of therapeutic activity of CPA7 in intracranial tumor models was most likely attributed to the poor diffusion of CPA-7 across the BBB. This was confirmed by IHC and immunoblotting of peripheral and intracranial tumors, in addition to results obtained with PAMPA. To this effect, platinum-based complexes are notorious for having poor brain permeability and show poor efficacy for treating CNS neoplasms (Gormley et al., 1981, Nakagawa et al., 1994). The success of many clinical trials fringes on the safety of the therapies in question at an efficacious dose. Our results for CPA-7 at the therapeutic dose showed normal liver tissue, normal sinusoids in the spleens of flank tumor-
bearing animals treated with the compound, and slight accumulation of protein and a flattening of the epithelial lining of the tubules in the kidneys, indicative of tissue regeneration in response to an injury. In order to fully understand and characterize the safety features of this compound and before clinical implementation, full toxicology assessment is warranted. In summary, CPA-7 constitutes a powerful anti-cancer agent in models of peripheral solid cancers. Our data strongly supports further development of CPA-7 derived compounds with optimum permeability features in order to enhance their efficacy in intracranial tumors, i.e., GBM.

Further our data demonstrates several important points, i.e., 1- It highlights the importance of testing chemotherapeutic drugs in vivo, as all compounds exhibited cytotoxicity when tested in rodent tumor cells, human tumor cells and human tumor stem cells in vitro; 2- It highlights the critical importance of testing drugs in appropriate in vivo models, which reproduce as closely as possible the human condition. Our data conclusively demonstrates that although CPA-7 shows efficacy when used to treat tumors located in the periphery, when the same tumor is located within the central nervous system, the compound does not exhibit anti-tumor activity. Further, we demonstrate that this is due to the lack of penetrance of the chemotherapeutic compound into the brain due to the presence of the blood brain barrier (Figure 5, Panels C, G, D, and H); 3- The data also highlights that thorough and comprehensive testing of novel compounds in the appropriate in vivo animal model is critical before attempting to translate these compounds into the clinical arena (van der Worp et al., 2010, Prinz et al., 2011, Martic-Kehl et al., 2012, Seok et al., 2013, Collins et al., 2014).
Acknowledgements

We are grateful for the insight and expertise provided by Drs. Richard Keep and Raoul Kopelman. We are also extremely thankful for the academic leadership and support received from Dr. Karin Murasko and the Department of Neurosurgery; to M. Dahlgren, D. Tomford, and S. Napolitan for superb administrative support; and to R. Lemons and M. Dzaman for outstanding technical assistance.
Authors’ Contributions

- Participated in research design: Assi, Paran, VanderVeen, Savakus, Doherty, Lowenstein, and Castro.
- Conducted experiments: Assi, Paran, VanderVeen, Savakus, Doherty, Petruzzella, Hoeschele, Appelman, Lowenstein, and Castro.
- Contributed new reagents or analytical tools: Petruzzella, Hoeschele, Raptis, and Mikkelsen.
- Performed data analysis: Assi, Paran, VanderVeen, Savakus, Doherty, Petruzzella, Hoeschele, Appelman, Lowenstein, and Castro.
- Wrote or contributed to the writing of the manuscript: Assi, VanderVeen, Doherty, and Castro.
References


Footnotes

**Financial Support:** This work was supported by National Institutes of Health/National Institute of Neurological Disorders & Stroke (NIH/NINDS) [Grants U01-NS052465, U01-NS052465-S1, R01-NS074387, R01-NS057711, MICHR Pilot R14 U040007]; and Biointerfaces Institute, University of Michigan [U042841] to M.G. C.; NIH/NINDS [Grants R01-NS054193, R01-NS061107, R01-NS082311, R21-NS084275, and M-Cube U036756] University of Michigan to P.R.L.; the Department of Neurosurgery, University of Michigan School of Medicine; the Michigan Institute for Clinical and Health Research, NIH [Grant UL1-TR000433]; University of Michigan Cancer Biology Training Grant, NIH/NCI (National Cancer Institute) [Grant T32-CA009676]; University of Michigan Training in Clinical and Basic Neuroscience, NIH/NINDS [Grant T32-NS007222]; the University of Michigan Medical Scientist Training Program, NIH/NIGMS (National Institute of General Medicine Sciences) [Grant T32-GM007863], and the National Institutes of Health through the University of Michigan’s Cancer Center Support [Grant P30-CA046592]. We acknowledge the funding received from the Biointerfaces Institute, University of Michigan U042841 to M.G.C. and M-Cube U036756 University of Michigan to P.R.L., and Mr. Phil F. Jenkins for his generous support toward the purchase of a Zeiss 3D Scanning Electron Microscope.
FIGURE LEGENDS

**Figure 1.** Small molecule inhibitors of STAT3 block the proliferation of rodent and human glioma cells *in vitro*. (A) Dose-response curves were generated by plating mouse (Gl26, SMA560), rat (CNS-1), and human (U251, IN859, HF2303) glioma cells in 96-well plates and adding CPA-7 (red), WP1066 (green), or ML116 (blue) at doses ranging from 10 nM to 100 µM. After 48 hours of treatment, ATP levels were measured in triplicate using the Cell-Titer Glo® kit and plotted versus drug concentration (*, *p* < 0.05 versus vehicle; two-way ANOVA followed by Tukey’s test). (B) IC₅₀ values for CPA-7, WP1066, and ML116 in mouse (Gl26, SMA560), rat (CNS-1), and human (U251, IN859, HF2303) glioma cell lines. (C) Proliferation assays were performed in a similar fashion but were cultured in the presence of a single dose of drug over multiple days. To assess proliferation, ATP levels were measured every day in triplicate over the course of 5-6 days. Growth of glioma cells was dramatically inhibited in response to CPA-7, WP1066, and ML116 to in the majority of cell lines tested (*, *p* < 0.05 versus vehicle; two-way ANOVA followed by Tukey’s test).

**Figure 2.** Inhibition of STAT3 in glioma cells elicits cell death. Glioma cells were cultured with STAT3 inhibitor at the dose indicated for a period of 48 hours in triplicate wells. Cells were analyzed by flow cytometry following staining with Annexin V and propidium iodide (PI) (A) Representative dot plots from flow cytometric analysis of mouse (Gl26, SMA560), rat (CNS-1), and human (U251, IN859, HF2303) glioma cell lines treated with Annexin V and PI presented on the x- and y-axis, respectively. (B) Cells deemed to be apoptotic (Annexin V+ or
AnnexinV+/PI+) were quantified and presented bar graphs. Inhibition by STAT3 small molecule inhibitors elicits significant levels of cell death (*, \( p < 0.05 \) versus vehicle; one-way ANOVA followed by Tukey-Kramer multiple-comparison test).

**Figure 3.** Decreased expression of STAT3 transcriptional targets is evident in response to small molecule inhibitors. (A, B, C) Mouse (Gl26, SMA560), rat (CNS-1), and human (U251, IN859, HF2303) glioma cell lines were treated with CPA-7 or WP1066 at the indicated doses for a period of 24 hours followed by lysis and electrophoretic separation of total proteins on a polyacrylamide gel. Proteins were transferred on a PVDF membrane and immunoblotted for pSTAT3, total STAT3, or their downstream targets. Levels of actin were used as a loading control.

**Figure 4.** Transcriptional specificity of STAT3 inhibitors. Transcriptional activity was assessed in triplicate and is represented as the normalized ratio of luciferase signal divided by a separate measurement of ATP levels account for cell viability. (A) STAT3-mediated luciferase activity was quantified after a 24 hour treatment with CPA-7, ML116, or WP1066 at the indicated doses (*, \( p < 0.05 \); one way ANOVA followed by Tukey’s test). (B) STAT3 inhibitors or MG132 (known NF-\( \kappa \)B inhibitor) were added 1 hour prior to the addition LPS. LPS was used to stimulate the NF-\( \kappa \)B pathway (†, \( p < 0.05 \); one-way ANOVA followed by Tukey’s test). NF-\( \kappa \)B activity was measured after a 6 hour LPS treatment period (*, \( p < 0.05 \); one-way ANOVA followed by Tukey’s test). (C) STAT5-mediated expression of luciferase was quantified after a 6 hour treatment period with Pimozide (known STAT5 inhibitor) or STAT3 inhibitors. (D) CPA-7, WP1066, ML116, and Nifuroxazide (known pan STAT inhibitor) were added 1 hour prior to the
addition of IFNγ, which was used to stimulate STAT1 activity. STAT1-specific luciferase expression was measured 6 hours later (*, \( p < 0.05 \); one-way ANOVA followed by Tukey’s test.

**Figure 5.** Therapeutic efficacy of CPA-7 in peripheral subcutaneous tumors and intracranial brain tumors. (A) 20,000 GL26 cells were implanted in the striatum of C57BL/6J mice. (B) Mice bearing brain tumors were treated 4 days later with CPA-7 intravenously with no discernible therapeutic effect as seen in the Kaplan-Meier curve (n=4, 5 mg/kg every three days for 15 days). (C) Mice bearing brain tumors were treated with three doses of CPA-7 (5 mg/kg) over a span of 7 days before being harvested for IHC. Paraffin sections were immunostained with an anti-pTyr705STAT3 antibody and visualized using DAB peroxidase. Micrographs were acquired using 5X and 40X objectives. (D) Intracranial tumors from a duplicate treatment cohort were harvested, homogenized, and lysed for western blot analysis. (E) C57BL/6J mice were injected with \( 1 \times 10^6 \) GL26 cells in the hind flank to generate subcutaneous tumors. (F) Mice bearing flank tumors were treated with CPA-7 (n=5, 5 mg/kg every three days for 15 days) when tumors reached an approximate volume of 30-60 mm\(^3\). Plotted values represent the means of tumor volumes ± SEM. (*, \( p < 0.05 \); extra sum-of-squares test). (G) Mice bearing GL26 flank tumors were treated three times with CPA-7 (5 mg/kg) over a span of 7 days before being harvested for IHC. Paraffin sections were immunostained with an anti-pTyr705STAT3 antibody and visualized using DAB peroxidase. Micrographs were acquired using 5X and 40X objectives. (H) Flank tumors from a duplicate treatment cohort were harvested, homogenized, and lysed for western blot analysis. CPA-7 was effective at reducing the levels of pSTAT3 visualized by IHC or western blot (*, \( p < 0.05 \); one-tail students t-test).
Figure 6. STAT3 inhibitors are effective at treating peripheral tumors. (A) 50,000 B16-f0 or 200,000 B16-f10 melanoma cells were injected in the striatum of C57BL/6J mice. Treatment was initiated with CPA-7 (n=5; 5 mg/kg intravenously every three days) or WP1066 (n=5; 40 mg/kg oral gavage 5 days on 2 days off) on day 4 and continued until animals became moribund. Error bars represent ± SEM; *, p < 0.05; extra sum-of-squares test. (B) B16-f0 flank tumor-bearing mice showed no notable liver toxicity in any animal following treatment with the small molecule STAT3 inhibitors. (C) While no toxicity was observed in the spleens of CPA-7-treated mice, obliteration of the sinusoids by infiltration of transformed lymphocytes was found in 67% of the WP1066-treated animals (n=3). (D) Hematological analysis shows a depression in several parameters following treatment with CPA-7, including red blood cells, reticulocytes, white blood cells, lymphocytes, and monocytes when compared with the vehicle. Grey boxes represent normal ranges for this strain.
Figure 2

A

GL26 mouse GBM

Mock

+200μM CPA-7

+100μM WP1066

+50μM ML116

Annexin-V

U251 mouse GBM

Mock

+10μM CPA-7

+3μM WP1066

+10μM ML116

Annexin-V

IN859 human primary GBM

Mock

+10μM CPA-7

+3μM WP1066

+20μM ML116

Annexin-V

SMA560 mouse GBM

Mock

+5μM CPA-7

+1μM WP1066

+4μM ML116

Annexin-V

CNS1 rat GBM

Mock

+10μM CPA-7

+1μM WP1066

+4μM ML116

Annexin-V

HF2303 human primary GBM

Mock

+10μM CPA-7

+3μM WP1066

+20μM ML116

Annexin-V

B

% Annexin V+ / PI+

Vehicle

ML116

CPA-7

WP1066

GL26

SMA560

U251

CNS1

IN859

HF2303

*
Pre-clinical characterization of signal transducer and activator of transcription 3 small molecule inhibitors for primary and metastatic brain cancer therapy


The Journal of Pharmacology and Experimental Therapeutics (JPET)

Supplementary Materials and Methods

Synthesis, Administration, and Validation of STAT3 small molecule inhibitors.

CPA-7 (fac-[Pt(NH₃)₂Cl₃NO₂]; M.W. 381.5) was synthesized according to a previously published report (Littlefield et al., 2008). The reaction product was purified of KCl contaminants by acetone purification. Briefly, the pale yellow solution was dried using a rotary evaporator at 25°C. The solid sample was then transferred to a 250 mL Erlenmeyer flask and 50 mL of reagent grade acetone was added to dissolve the crude CPA-7 product. KCl, which remained undissolved, was removed by filtering through a medium-porosity filter into a single-neck round-bottom flask. The acetone was evaporated under vacuum and the residue was then dried under pressure then vacuum for 2 hours at 45°C.

The purity of the recrystallized product was assayed by HPLC (using an ICS-5000 ThermoFisher system) and determined to be 97.48% pure. Analysis of the product by ¹H-NMR (in acetone-d6) shows a multplet (septet) centered at 5.760 ppm, consistent with cis-diammines bonded to a Pt(Mora, #7) center in this asymmetric Pt(Mora, #7) complex.
Sensitivity to light and stability in darkness was also assessed for CPA-7 (Supplementary Figure 1). The UV spectrum of CPA-7 features an absorption maximum at a wavelength of 264 nm. As a measure of the stability of CPA-7, the absorbance of a solution of CPA-7 (6.0 mg/100 mL H₂O) was monitored at 264 nm over a period of 7 days. (After the solution was prepared, it was divided in half: 50 mL exposed to light & 50 mL kept in the dark). The data illustrate the changes in the absorbance as a function of time. It is clear that CPA-7 is sensitive to light and thus stock solutions must be protected from light to minimize changes.

Aliquots of 20 mM CPA-7 were prepared at in a 50:50 mixture of H₂O and DMSO, wrapped in foil (CPA-7 is light sensitive) and stored at -80°C long-term. Prior to in vivo administration of CPA-7, 20 mM (7.63 mg/mL) aliquots prepared in a 50:50 mixture of DMSO and H₂O were thawed and diluted with serum-free DMEM to the desired concentration (3.28 mM or 1.25 mg/mL). For intracranial and flank tumor survival studies, mice were administered 100 µL (5 mg/kg or 0.125 mg) of the diluted CPA-7 solution via tail vein injection. Six injections of CPA-7 were administered 3 days apart and were initiated 4 days post-implantation of intracranial tumors, and 25 days post-implantation of flank tumors.

Synthesis of WP1066 (C₁₇H₁₄BrN₃O; M.W. 356.2) was carried out by the Vahlteich Medicinal Chemistry Core at the University of Michigan according to the synthetic route outlined in Supplementary Figure 2a (Hussain et al., 2007).

Analytical HPLC was performed on an Agilent 1100 Series system with an Agilent Eclipse plus C18 (4.6 + 7.5 mm, 3.5 mm particle size) column (Supplementary Figure 2b). The mobile phase was a binary gradient of acetonitrile (containing 0.1 % TFA) and water (10–90%). Mass spectra were recorded on a Micromass LCT time-of-flight instrument utilizing the
electrospray ionization mode (Supplementary Figure 2c). $^1$H NMR spectra were recorded on a Varian 400 instrument at 400 MHz (Supplementary Figure 2d).

Aliquots of 10 mM WP1066 were prepared in DMSO and stored at -80°C long-term. Prior to in vivo administration of the drug, WP1066 aliquots were thawed and diluted to 10 mg/mL with PEG300 to make a final solution that is 80% PEG300 and 20% DMSO. For intracranial and flank tumor survival studies, 100 µL of 10 mg/mL WP1066 solution (40 mg/kg) was administered daily via oral gavage for 5 days, followed by 2 days of rest. Treatment of intracranial tumor-bearing mice began 4 days after tumor implantation, while treatment for flank tumors commenced 25 days after tumor implantation and continued until the animals showed signs of morbidity.

ML116 (C$_{18}$H$_{19}$N$_3$S; M.W. 309.4) was purchased from Ryan Scientific, Inc. and solubilized in DMSO as per the original synthetic route (Madoux et al., 2010). 10 mM aliquots were prepared for long-term storage at -80°C. For in vivo delivery of ML116, 16 mg/mL aliquots prepared in pure DMSO then diluted 1:4 in a carrier solution consisting of 30% hydroxypropyl-β-cyclodextrin and 25% PEG300 (final concentration of ML116 in the solution was 4 mg/mL). Mice bearing intracranial tumors were treated with ML116 (15 mg/kg) daily by intraperitoneal injection. Animals were treated with ML116 until they began to display symptoms of morbidity.

**Tumor models**

Female C57BL/6J mice (6-12 weeks) were anesthetized by IP injection of Ketamine (75 mg/kg) and Medetomidine (0.5 mg/kg). Once fully anesthetized, the mice were mounted onto a stereotactic frame and a small burr hole was drilled in the skull using a hand dremel. 20,000 GL26, 3,000 B16-f10, or 1,000 B16-f0 cells in 1 µl of PBS were injected unilaterally into the right striatum (+0.5 mm AP, +2.2 mm ML, -3.0 mm DV from bregma) using a 5µl Hamilton
syringe fitted with a 33-gauge needle. The needle was left in place for 3 minutes following the injection before being withdrawn slowly. The injection site was washed with sterile saline and the incision was closed using nylon sutures. Mice were resuscitated using atipamezole (Alonzi, #15) and administered buprinex (SQ) as an analgesic. To generate peripheral flank tumors 1x10^6 GL26, 2x10^5 B16-f10, or 5x10^4 B16-f0 were prepared in 100 µl of PBS and injected in the hind flanks of C57BL/6J mice. Using a hand caliper, tumor volume was estimated by measuring the width and length of the tumor mass and applying the formula $v = \frac{1}{2} (l \times w^2)$ where w is the smaller of two measurements. All animals were housed in specific pathogen free environment, and monitored closely. Animal tissues requiring histopathology were perfused using oxygenated, heparinized Tyrode’s solution (132 mM NaCl, 1.8 mM CaCl2, 0.32 mM NaH2PO4, 5.56 mM glucose, 11.6 mM NaHCO3, and 2.68 mM KCl) and perfused-fixed with 4% paraformaldehyde, respectively.

**Luciferase assay for transcription factor activity.**

U251 human glioma cells were transfected with the following plasmids to generate stable clones: pGL4.32[luc2P/NF-κβ-RE/Hygro], pGL4.52[luc2P/STAT5-RE/Hygro]. After selection with antibiotic, clones were screened and selected based on their luciferase activity. MG-132 (Sigma, St. Louis, MO, USA), Pimozide (Sigma, St. Louis, MO, USA), and nifuroxazide were used as inhibitors of NF-κβ, STAT5, and STAT1 respectively. LPS (100 ng/mL) and IFNγ (100 pg/mL) were used to stimulate NF-κβ and STAT1 activity, respectively. Firefly luciferase activity was measured using Promega’s dual luciferase assay kit. 30,000 U251 cells were plated in 12 well plates (Fisher, Waltham, MA, USA) in Complete DMEM. On day 2, CPA-7, ML116, and WP1066 were added to the appropriate wells at the indicated final concentrations. NF-κβ or STAT1 activity was stimulated with their appropriate cytokines 1.5 hours after addition of
inhibitor. 6 hours after LPS addition or incubation with STAT3 inhibitor, the wells were rinsed with PBS and lysed in 150 μL of Promega Passive Lysis Buffer (Promega), shaken for 20 minutes. Seventy-five μL of each well was then transferred to an opaque 96-well plate for measurement with 100 μL of Luciferase Assay Reagent (Promega) for 10 seconds in a Veritas Microplate Luminometer (Turner Biosystems). In a separate treatment plate, effects on cell viability were monitored by measuring ATP levels and described earlier.

**Western Blot**

Whole cell extracts were prepared by lysing the cells with RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM each NaF, NaVO4, and EGTA; 1% Igepol; 0.25% sodium deoxycholate; 1x protease/phosphotase inhibitors (Thermo Scientific, Waltham, MA) for 5 min on ice, then centrifuging at 12,000g for 10 minutes at 4°C to remove cell debris. Protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) and 15-30 μg of protein was denatured and reduced before loading onto a 4-12% SDS-Polyacrylamide gradient gel. Samples were transferred onto PVDF membranes for immunoblotting. Membranes were blocked with 5% milk in TBS+0.05% Tween20 and incubated with primary antibodies at the manufactures suggested dilution overnight at 4°C. Membranes were washed 3 times in TBS with 0.05% Tween20 and incubated with the appropriate HRP-conjugated secondary (Dako, Carpinteria, CA). Immunoreactivity was visualized using the SuperSignal West Femto substrate solution (Thermo Scientific) and imaged on a ChemiDoc MP imaging system (BioRad, Hercules, CA). Band intensity was digitally quantified using the imaging software which accompanies the ChemiDoc MP imaging system.
**Paraffin IHC**

Tumor specimens were fixed by perfusing with a 4% solution of paraformaldehyde, processed and embedded in paraffin for sectioning. 5 µM microtome sections of flank tumors, brain tumors, liver, kidney, and spleen were cut, mounted on a slide, deparaffinized and rehydrated for IHC or H&E. Tissue slides for IHC were placed in an antigen retrieval solution (1 mM EDTA, 10 mM Tris pH 9.0) and heated inside a pressure cooker to expose antigens. Sections were washed and permeabilized with TBS-TritonX100 (TBS-Tx 0.05%) solution. To inactivate endogenous peroxidases, a solution of 0.3% H$_2$O$_2$ /PBS was added to the section and allowed to sit for 20 minutes at room temperature before proceeding with a blocking step (TBS-Tx with 10% normal goat serum for 1 hour at room temperature). After blocking, primary antibody was added to the sections at the manufacturer’s suggested dilution and allowed to bind at 4°C overnight. The following day, sections were washed five times with TBS-Tx before adding the appropriate biotinylated secondary antibody. After secondary incubation, sections were washed five times with TBS-Tx before the addition of Vectastain ABC solution (Vector Labs, Burlingame, CA), which contains the avidin/biotin complex. Chromogenic detection of peroxidase was detected using DAB as an enzyme substrate. Sections were then dehydrated and coverslipped for imaging on a Zeiss Axioplan 2 microscope.

**CBC and Serum Chemistry**

CBC was performed on an IDEXX Procyte Dx Hematology Analyzer within 15 minutes of blood draw according to the manufacturer’s instructions. Serum chemistry was performed on a VetTest 8008 Chemistry Analyzer according to the manufacturer’s instructions.
Hematoxylin & Eosin Histology

The livers, kidneys, and spleens were post-fixed for 2 days in 4 % paraformaldehyde prior to dehydration on a Leica ASP 300 processor, embedded on a Tissue-Tek embedding station, and sectioned on a Leica RM 2135 microtome at 5 μm. Liver, kidney, and spleen sections were stained with Hematoxylin and Eosin. Slides were analyzed at the Department of Pathology at the University of Michigan and imaged on a Zeiss Axioplan 2 microscope.
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Quality control for CPA-7 synthesis. (a) Chromatogram of HPLC performed on the purified synthesized product indicates the presence of a single compound with trace contaminants (97.5% purity). (b) Stability of CPA-7 over time when kept in the dark. (c) Light sensitivity of CPA-7.

Supplementary Figure 3. Synthesis and quality control measurements for WP1066. (a) Proposed synthetic route for WP0166. (b) HPLC data indicates high purity of a single compound (>99.7%). (c) Positive ion mass spectrometry analysis showing the ions produced from the fragmentation of the synthesized product. Peaks at 356 m/z indicate presence of a compound with a molecular mass equivalent to that of WP1066. (d) $^1$H-NMR spectrum conforms to the predicted molecular structure of WP1066.

Supplementary Figure 3. Immunoblot for pSTAT3 against glioma and melanoma cells. Expression of phosphorylated STAT3 is variable amongst glioma cell lines. Whole cell lysates were obtained from cultured tumor cells using RIPA buffer. Equal amounts of total clarified protein were separated on an SDS polyacrylamide gel and immunoblotted with antibodies against pSTAT3, total STAT3, and actin.

Supplementary Figure 4. Inhibition of STAT3 phosphorylation in response to ML116 treatment. U251 human glioma cells were treated with increasing doses of ML116 before undergoing lysis. Equivalent amounts of clarified whole cell lysate were analyzed by Western blot using antibodies against pTyr705STAT3, total STAT3, and actin. Inhibition of STAT3 was achieved at a high dose of ML116 (100 µM).
Supplementary Figure 5. Therapeutic efficacy of WP1066 and ML116 in mice bearing GL26 tumors. (a) Schematic showing the experimental approach to intracranial GL26 tumor implantation and treatment strategies. Mean survival time of C57BL/6J mice implanted with 20,000 GL26 tumor cells and treated 4 days after tumor implantation with (b) WP1066 (40 mg/kg; oral gavage, 5 days “on,” and 2 days “off”) or (c) ML116 (15 mg/kg; I.P., daily) was similar to vehicle treated controls. (d) Schematic showing the experimental approach to GL26 flank tumor implantation and treatment strategies. Similarly, C57BL/6J mice implanted with 1x10^6 GL26 cells in the hind flank and treated 25 days after tumor implantation with (e) WP1066 (40 mg/kg; oral gavage, 5 days “on,” and 2 days “off”) or (f) ML116 (15 mg/kg; I.P., daily) showed no statistically significant difference in the growth of peripheral GL26 tumors.

Supplementary Figure 6. Passive permeability of STAT3 inhibitors using Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA was used to measure the permeability of drugs across a lipid membrane. Briefly, compounds were added to the bottom chamber of donor plates at a concentration of 50 µM. A membrane was coated with a lipid solution and sandwiched between the donor and acceptor plates. The plates were incubated for 4 hours before measuring the passive diffusion across chambers with UV absorption. The effective permeability (cm/s) or P_{eff} was derived with the software provided by Pion, Inc.

Supplementary Figure 7. Treatment of intracranial melanoma tumors with WP1066 and ML116. 1,000 B16-f0 or 1,000 B16-f10 melanoma cells were injected in the striatum of C57BL/6J mice. Treatment was initiated with CPA-7 (n=5; 5 mg/kg, IV, every three days),
ML116 (n=5; 20 mg/kg, IP, every day), or WP1066 (n=5; 40 mg/kg, oral gavage, 5 days “on,” and 2 days “off”) on day 4 and continued until animals became moribund.

Supplementary Figure 8. Toxicity in the kidneys of B16-f0 melanoma flank tumor-bearing mice treated with STAT3 small molecule inhibitors. Kidneys featured normal tissue for both the vehicle- and WP1066-treated animals in the central cortexes and subcapsular tubules. In the CPA-7-treated animals (n=3), however, protein was noted in many of the tubules of the central cortex (a) accompanied by flattened epithelium surrounding these tubules (b), characteristic of tissue regeneration as a result of an injury. Within the subcapsular tubules, CPA-7-treated mice revealed relatively smaller cells with darker cytoplasm (c), also indicating injury.

Supplementary Figure 9. Treatment of flank B16-f10 melanoma tumors with ML116. 200,000 B16-f10 melanoma cells were injected subcutaneously in the flanks of C57BL/6J mice. Treatment with ML116 began 25 days after tumor implantation (20 mg/kg, IP, every day) showed no significant difference in growth kinetics when compared to the vehicle.
Supplementary Figure 1. Quality control for CPA-7 synthesis. A. Chromatogram of HPLC performed on the purified synthesized product indicates the presence of a single compound with trace contaminants (97.5% purity). B. Stability of CPA-7 over time when kept in the dark. C. Light sensitivity of CPA-7.
Supplementary Figure 2. Synthesis and quality control measurements for WP1066.

A. Proposed synthetic route for WP0166. B. HPLC data indicates high purity of a single compound (>99.7%). C. Positive ion mass spectrometry analysis showing the ions produced from the fragmentation of the synthesized product. Peaks at 356 m/z indicate presence of a compound with a molecular mass equivalent to that of WP1066. D. ¹H-NMR spectrum conforms to the predicted molecular structure of WP1066.
Supplementary Figure 3. Immunoblot for pSTAT3 against glioma and melanoma cells. Expression of phosphorylated STAT3 is variable amongst glioma cell lines. Whole cell lysates were obtained from cultured tumor cells using RIPA buffer. Equal amounts of total clarified protein were separated on an SDS polyacrylamide gel and immunoblotted with antibodies against pSTAT3, total STAT3, and actin.
Supplementary Figure 4. Inhibition of STAT3 phosphorylation in response to ML116 treatment. U251 human glioma cells were treated with increasing doses of ML116 before undergoing lysis. Equivalent amounts of clarified whole cell lysate were analyzed by Western blot using antibodies against pTyr705STAT3, total STAT3, and actin. Inhibition of STAT3 was achieved at a high dose of ML116 (100 µM).
Supplementary Figure 5. **Therapeutic efficacy of WP1066 and ML116 in mice bearing GL26 tumors.**

**A.** Schematic showing the experimental approach to intracranial GL26 tumor implantation and treatment strategies. **B.** Mean survival time of C57BL/6J mice implanted with 20,000 GL26 tumor cells and treated 4 days after tumor implantation with WP1066 (40 mg/kg; oral gavage, 5 days “on,” and 2 days “off”). **C.** Therapeutic efficacy of ML116 against intracranial GL26 challenge (15 mg/kg; I.P., daily) **D.** Schematic showing the experimental approach to GL26 flank tumor implantation and treatment strategies. **E.** C57BL/6J mice were implanted with 1x10^6 GL26 cells in the hind flank and treated 25 days after tumor implantation with WP1066 (40 mg/kg; oral gavage, 5 days “on,” and 2 days “off”). **F.** ML116 administration (15 mg/kg; I.P., daily) to tumor bearing mice demonstrating poor tumoricidal efficacy.
**Supplementary Figure 6.** Passive permeability of STAT3 inhibitors using Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA was used to measure the permeability of drugs across a lipid membrane. Briefly, compounds were added to the bottom chamber of donor plates at a concentration of 50 µM. A membrane was coated with a lipid solution and sandwiched between the donor and acceptor plates. The plates were incubated for 4 hours before measuring the passive diffusion across chambers with UV absorption. The effective permeability (cm/s) or $P_{\text{eff}}$ was derived with the software provided by Pion, Inc.
Supplementary Figure 7. Treatment of intracranial melanoma tumors with WP1066 and ML116. 1,000 B16-f0 or 1,000 B16-f10 melanoma cells were injected in the striatum of C57BL/6J mice. Treatment was initiated with CPA-7 (n=5; 5 mg/kg, IV, every three days), ML116 (n=5; 20 mg/kg, IP, every day), or WP1066 (n=5; 40 mg/kg, oral gavage, 5 days “on,” and 2 days “off”) on day 4 and continued until animals became moribund.
Supplementary Figure 8. Toxicity in the kidneys of B16-f0 melanoma flank tumor-bearing mice treated with STAT3 small molecule inhibitors. Kidneys featured normal tissue for both the vehicle- and WP1066-treated animals (in the central cortexes and subcapsular tubules). In the CPA-7-treated animals (n=3), however, protein was noted in many of the tubules of the central cortex (a) accompanied by flattened epithelium surrounding these tubules (b), characteristic of tissue regeneration as a result of an injury. Within the subcapsular tubules, CPA-7-treated mice revealed relatively smaller cells with darker cytoplasm (c), also indicating injury.
Supplementary Figure 9. Treatment of flank B16-f10 melanoma tumors with ML116. 200,000 B16-f10 melanoma cells were injected subcutaneously in the flanks of C57BL/6J mice. Treatment with ML116 began 25 days after tumor implantation (20 mg/kg, IP, every day) showed no significant difference in growth kinetics when compared to the vehicle.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA-7</td>
<td>-Good Solubility -Selective for STAT3 -High doses of CPA-7 can block STAT1 and STAT5 -Low IC50; 1-10µM -Blocks STAT3 DNA binding</td>
<td>18, 19, 20, 21</td>
</tr>
<tr>
<td>WP1066</td>
<td>- Inhibits upstream kinase JAK2 -Low IC50; 0.5-10µM -Nonselective for STAT3</td>
<td>12, 23, 24, 26, 38</td>
</tr>
<tr>
<td>ML116</td>
<td>-Newly identified compound -Inhibits STAT3 DNA binding -Poor Solubility: 1.6µM in PBS -High IC50</td>
<td>25</td>
</tr>
</tbody>
</table>

Supplementary Table 1. STAT3 small molecule inhibitor characterization. The structure, physical properties, and specificity within the JAK-STAT pathway are shown.
Supplementary Table 2. Systemic toxicity in flank tumor-bearing mice following treatment with STAT3 inhibitors, i.e., WP1066 or CPA-7. Hematological and biochemical parameters are shown for mice bearing flank tumors with mean values and SEM (Standard Error of the Mean).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reference Range</th>
<th>Vehicle</th>
<th>WP1066</th>
<th>CPA-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (Red Blood Cells; M/μL)</td>
<td>7.4 - 12</td>
<td>5.85</td>
<td>6.71</td>
<td>3.57</td>
</tr>
<tr>
<td>HGB (Hemoglobin; g/dL)</td>
<td>10.8 - 19.2</td>
<td>8.58</td>
<td>9.58</td>
<td>5.13</td>
</tr>
<tr>
<td>HCT % (Percent Hematocrit)</td>
<td>37.3 - 62</td>
<td>29.40</td>
<td>34.03</td>
<td>15.27</td>
</tr>
<tr>
<td>RET (Reticulocytes; K/μL)</td>
<td>2.4 - 10.6</td>
<td>542.35</td>
<td>686.83</td>
<td>10.62</td>
</tr>
<tr>
<td>RET % (Percent Reticulocytes)</td>
<td>2.2 - 6.4</td>
<td>16.15</td>
<td>17.53</td>
<td>0.17</td>
</tr>
<tr>
<td>Ptt (Platelet; K/μL)</td>
<td>840 - 2200</td>
<td>646.00</td>
<td>649.25</td>
<td>638.67</td>
</tr>
<tr>
<td>WBC (White Blood Cells; K/μL)</td>
<td>3.9 - 14</td>
<td>2.81</td>
<td>2.29</td>
<td>0.78</td>
</tr>
<tr>
<td>Neutrophils (K/μL)</td>
<td>0.4 - 3</td>
<td>0.36</td>
<td>0.41</td>
<td>0.31</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>7.3 - 28.6</td>
<td>14.83</td>
<td>16.10</td>
<td>33.27</td>
</tr>
<tr>
<td>Lymphocytes (K/μL)</td>
<td>2.8 - 11.2</td>
<td>2.12</td>
<td>1.75</td>
<td>0.40</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td>61 - 87</td>
<td>75.63</td>
<td>78.45</td>
<td>59.10</td>
</tr>
<tr>
<td>Monocytes (K/μL)</td>
<td>0.15 - 0.94</td>
<td>0.31</td>
<td>0.10</td>
<td>0.03</td>
</tr>
<tr>
<td>Monocyte %</td>
<td>2 - 11</td>
<td>8.20</td>
<td>4.03</td>
<td>3.80</td>
</tr>
<tr>
<td>Eosinophils (K/μL)</td>
<td>0.01 - 0.5</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>0.013 - 4.5</td>
<td>1.05</td>
<td>1.18</td>
<td>3.83</td>
</tr>
<tr>
<td>Basophils (K/μL)</td>
<td>0 - 0.14</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Basophils %</td>
<td>0.01 - 1.24</td>
<td>0.15</td>
<td>0.25</td>
<td>0.00</td>
</tr>
<tr>
<td>ALB (Albumin; g/dL)</td>
<td>2.6 - 3.8</td>
<td>1.40</td>
<td>1.75</td>
<td>1.97</td>
</tr>
<tr>
<td>ALKP (Alkaline Phosphatase; U/L)</td>
<td>35 - 96</td>
<td>51.50</td>
<td>55.50</td>
<td>73.33</td>
</tr>
<tr>
<td>ALT (Alanine Aminotransferase; U/L)</td>
<td>28 - 129</td>
<td>45.25</td>
<td>67.00</td>
<td>41.00</td>
</tr>
<tr>
<td>Ca (Calcium; mg/ml)</td>
<td>110 - 129</td>
<td>9.63</td>
<td>9.63</td>
<td>-</td>
</tr>
<tr>
<td>CREA (Creatinine; mg/dL)</td>
<td>0.2 - 0.5</td>
<td>0.23</td>
<td>0.15</td>
<td>0.47</td>
</tr>
<tr>
<td>PHOS (Phosphorus; mg/dL)</td>
<td>7.9 - 14.5</td>
<td>12.83</td>
<td>1.15</td>
<td>-</td>
</tr>
<tr>
<td>TBIL (Total Bilirubin; mg/dL)</td>
<td>0.2 - 0.6</td>
<td>0.18</td>
<td>0.20</td>
<td>0.17</td>
</tr>
<tr>
<td>TP (Total Protein; g/dL)</td>
<td>4.8 - 7</td>
<td>3.60</td>
<td>4.25</td>
<td>4.40</td>
</tr>
<tr>
<td>BUN (Blood Urea Nitrogen; mg/dL)</td>
<td>7 - 28</td>
<td>30.50</td>
<td>19.50</td>
<td>84.33</td>
</tr>
<tr>
<td>GLOB (Globulin; g/dL)</td>
<td>2.18</td>
<td>2.40</td>
<td>2.43</td>
<td>1.88</td>
</tr>
</tbody>
</table>